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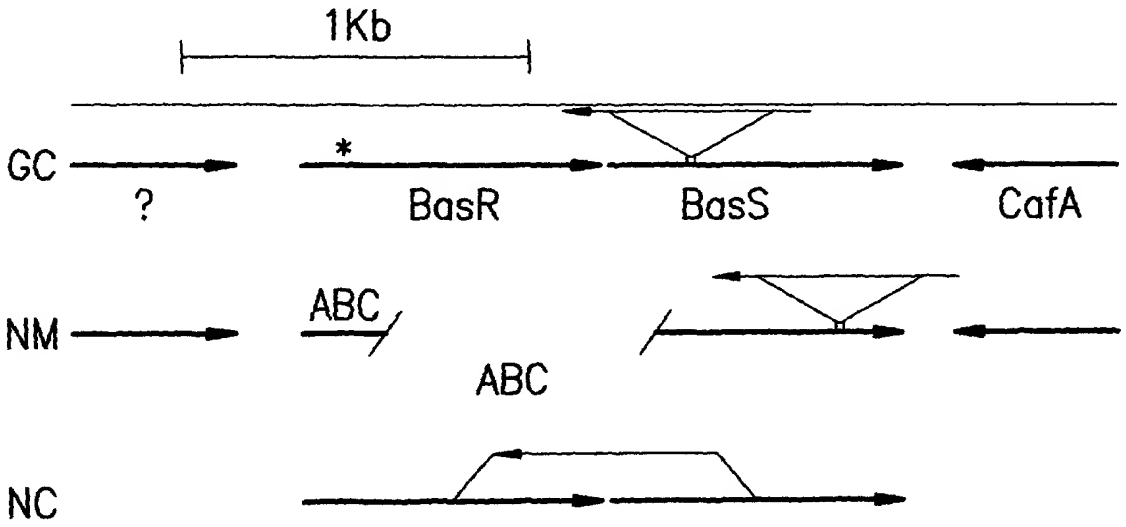
(54) Title: CONTROL OF NEISSERIAL MEMBRANE SYNTHESIS



(57) Abstract: The present invention discloses a mutant *Neisseria* having extensive membrane blebbing, both an indicium and a cause of virulence in the gonococcus and meningococcus. Methods are disclosed for making and characterizing the mutant, *bmrRS*. Methods are disclosed for isolating *bmrRS* membranes for use as a vaccine. Methods are also disclosed for identifying proteins that are unique to the mutant *Neisseria*. Proteins coded for or under the control of the *bmr* locus are provided. Methods are also disclosed for the use of the unique mutant proteins for determining the virulence of clinical samples of *N. gonorrhoeae* and *N. meningitidis*. Methods are also disclosed for the screening of antibiotics targeted to virulent *Neisseria*.

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CONTROL OF NEISSERIAL MEMBRANE SYNTHESIS

Background of the invention

Neisseria gonorrhoeae ("gonococcus") and *N. meningitidis* ("meningococcus") are Gram-negative diplococci that are strictly human pathogens. *N. gonorrhoeae* causes primarily urethritis in males and pelvic inflammatory disease in females. *N. meningitidis* is the causative agent of middle ear infections and meningitis.

As is commonly found in other bacterial pathogens, strains of *Neisseria sp.* vary greatly in pathogenicity. Treatment and outcome of neisserial infections are dictated by pathogenicity. For example, *N. meningitidis* is frequently found in the throat of normal humans, where it can reside as a commensal without causing symptoms. However, virulent strains of *N. meningitidis* may cause a fulminating meningitis, resulting in brain damage or death before treatment can control the disease. Diagnostic methods to date have not been useful in distinguishing the mild, commensal *Neisseria meningitidis* from the virulent strains.

The standard method of diagnosing a bacterial disease is culturing, followed by identification by immunoreactivity, morphology, and biochemical reactions. In the case of neisserial pathogens, because of its fastidious growth requirements, the organism has often lost viability and will no longer grow in culture once isolated from the patient. United States Patent Number 4,446,230 discloses a test method and bacterial strain for the laboratory diagnosis of gonorrhea. This strain can be maintained in a laboratory and will become transformed by exogenous neisserial DNA, even from a non-viable clinical sample. The transformation corrects an induced specific growth requirement, thereby permitting the strain to grow. However, this test does not distinguish virulent from non-virulent strains.

In vivo models of *N. gonorrhoeae* infection using male human volunteers have been done to elucidate the factors that contribute to bacterial virulence. However, such studies are costly and limited in scope. Recent tissue culture

models of gonococcal infection have begun to define the interactions between the bacterium and the host. In brief, it is evident that the bacteria attach and invade human urethral epithelial cells, a niche that probably represents the primary site of infection during the course of urethritis. Gonococci reside and replicate within vacuoles within these cells. Infected cells can rupture or be shed into the urethral lumen, releasing gonococci to invade neighboring epithelial cells or to be excreted in the urine. Several gonococcal components, including pili, Opa proteins and lipooligosaccharide (LOS) have been implicated in the ability of gonococcus to attach to and invade host cells, and implicated in the pathogenesis of gonococcal infection.

Our current knowledge clearly suggests that the gonococcus exists within different environments during the course of infection, such as extra- and intra-cellular and vacuolar locations. It is also likely that the site of infection of *N. gonorrhoeae*, the genital tract, differs significantly between males and females. How *N. gonorrhoeae* adapts to these different conditions is not well understood. It has been observed that a gonococcus thought to be pathogenic quickly converts to a less virulent type under the conditions of culture that are used to identify the bacterium and to test for antibiotic sensitivity.

Current treatment of neisserial infections is with broad spectrum antibiotics. However, treatment with broad spectrum antibiotics leads to the disturbance of the natural microflora, leaving the patient susceptible to infections with such opportunistic pathogens as *Candida albicans* and *Gardnerella*. An antibiotic targeted specifically at pathogenic, rather than commensal *Neisseria*, would avoid this complication. It is currently difficult to test virulent clinical isolates of *Neisseria* to determine each strain's sensitivity to targeted-specificity antibiotics because of the difficulty of maintaining virulence during growth of the strains in culture. Therefore, such an antimicrobial sensitivity test as is disclosed in United States Patent Number 5,789,173 may not be useful in determining neisserial sensitivity to antibiotics.

If the factors that convert a commensal, mildly or non-pathogenic *Neisseria meningitidis* or *N. gonorrhoeae* into a pathogenic, invasive bacterium were known, it would be possible to use the identification of such factors as an aid for the diagnosis and therapy of neisserial disease. There is a need to

determine these so that they can be used as a target in screening bacteriostatic or bacteriocidal drugs that are selectively effective against virulent *Neisseria.*

Brief Summary of the Invention

The present invention provides a mutant *Neisseria* bacterium having excessive outer membrane. The bacterium may be *Neisseria gonorrhoeae* or *Neisseria meningitidis.*

The present invention also provides a method of identifying a pathogenic bacterium comprising culturing under identical conditions 1) a mutant *Neisseria* bacterium having excessive outer membrane, 2) a wild type *Neisseria* and 3) a clinical sample taken from a patient suspected of having a neisserial infection; examining the extent of blebbing in each cultured bacterium; and comparing the extent of blebbing of the clinical sample to that to the bacterium having excessive outer membrane and to that of the wild type *Neisseria* so as to determine the pathogenicity of the clinical sample.

The present invention further provides a method of identifying an inhibitor compound of virulent *Neisseria* providing a culture containing a bacterium having a mutation which results in blebbing of the outer membrane and the test inhibitor compound, and providing a culture containing a wild type bacterium having no blebbing and the test inhibitor; and comparing the growth of the mutant bacterium to that of the wild type in order to identify an inhibitor compound that inhibits the growth of the mutant to a greater degree than the inhibitor compound inhibits the growth of the wild type.

The present invention further provides individual proteins coded for or otherwise under the control of the two-component system, the presence of which quickly and easily determined and is indicative of virulence.

Description of the Drawings

Figure 1 shows a diagram of the *bmr* locus of *N. gonorrhoeae* (NG), *N. meningitidis* (NM) and *N. cinerea* (NC).

Figure 2 shows the alignment of the amino acid sequences of the BmrR BmrS open reading frames (ORF) from *N. gonorrhoeae* (GC), *N. meningitidis* (NM) and *N. cinerea* (NC).

Figure 3 shows scanning electron micrographs of wild type and mutant *N. gonorrhoeae.*

Figure 4 shows scanning electron micrographs of wild type and mutant *N. meningitidis*.

Figure 5 shows scanning electron micrograph showing a comparison of *N. gonorrhoeae* strain 1291 and the superblebber *N. Gonorrhoeae* 1291*bmrS* mutant.

Figure 6 shows a radioautography of two dimensional gels showing differences in protein expression in *N. Gonorrhoeae* strain 1291 and the superblebber *N. Gonorrhoeae* 1291*bmrS* mutant cultured under identical conditions.

Figure 7 shows a radiograph of two dimensional gels showing proteins *atpC, etfB, gpm, minD and nusG.*

Detailed Description of the Invention

Neisseria gonorrhoeae and *N. meningitidis* are strictly human pathogens. Current knowledge of the pathogenesis of these organisms suggests that these bacteria encounter different environments within the host during infection. However, little is known of how *Neisseria* sense and respond to these different environments or what systems contribute to virulence. Many bacteria respond to changes in their environment through changes in the expression level of particular genes. Two-component regulatory systems are now recognized as significant mediators of signaling in bacteria, relaying environmental signals that produce changes in gene expression patterns. During signal transduction by a typical two-component regulator, a membrane sensor protein detects a specific environmental stimulus presumably either through a direct interaction with a ligand or through conformational changes induced by changes in environmental conditions. Such a regulatory system might be useful as a diagnostic marker of virulence in *Neisseria*.

However, information regarding signaling within the gonococcus is limited. Previously a two-component regulator-like system was reported in pilus gene expression, although the environmental cue to which the system responds is unknown. Since membrane blebbing has been found to be associated with virulence, it was thought that a quantitative assay of the amount of membrane would be indicative of increased virulence. In order to further investigate a possible two-component regulator-mediated signaling pathway in

N. gonorrhoeae, the polymerase chain reaction (PCR) with degenerate oligonucleotide primers was used to amplify a region from the DNA-binding component of a novel *N. gonorrhoeae* two-component regulator.

This regulatory system is known to be present in other *Neisseria* species and to have a role in outer-membrane blebbing. The term "blebbing" is used to mean the bubble- or blister-like extrusion of membrane from the surface of the bacterial cell. The term "high-blebbing" is used to describe bacteria showing such extensive outer membrane formation. "Blebs" are easily separated from the outer membrane by mild physical manipulation, such as differential centrifugation.

Outer membrane bleb formation by *Neisseria meningitidis* and *N. gonorrhoeae* has long been recognized as an important factor in *Neisseria* infection, particularly that due to *N. meningitidis*. Numerous membrane blebs were found at the tissue surface during ultrastructural analysis of cervical gonorrhoea (Evans et al. J. Infect. Dis. (1977) 136:248-255) and a meningococcus recovered from the plasma of an infected patient contained multiple, long membranous protrusions typical of blebbing (Brandtzaes et al. J. Infec. Dis. (1989) 155:195-204). Lipooligosaccharide (LOS), which is a component of the blebs shed by the gonococcus and meningococcus, has major physiological effects during sepsis and meningitis caused by *N. meningitidis* and the plasma LOS levels are closely correlated to prognosis. Furthermore, compartmentalization of LOS production correlates with the clinical presentation in meningococcal infection. LOS levels in patients defined as having septicaemia showed high levels in plasma (median 3500 ng/ml) and low levels in cerebral spinal fluid, while with patients with meningitis, LOS was detectable in the plasma of three out of 19 patients and in the CSF in 18 of 19 patients, with median levels of 2500 ng/ml.

From these observations, it is possible that blebbing is both the indicium and the cause of pathogenicity in *Neisseria*. Agents that control or inhibit blebbing may be useful in therapy against *Neisseria* infections. However, the standard laboratory cultures of *Neisseria* typically show little or no blebbing after repeated laboratory passage and are therefore not useful in screening for agents that control or inhibit blebbing. Identification of the degree of blebbing

of newly isolated strains from clinical samples may be an early indication of the virulence of the pathogen and whether immediate, aggressive therapy is necessary.

Factors affecting the blebbing process are poorly understood. Strains of meningococcal serogroup A, B and C release membrane blebs in the log phase of growth but not in the lag phase, suggesting some form of regulation. The identification of a putative regulatory system, the mutation of which produces clear differences in the blebbing process is potentially of great interest to improvement of therapeutic strategies for treatment of *Neisseria* infections in which blebbing is a serious complication.

During the course of investigation, mutation of the genes controlling blebbing was successful, and after insertion into a strain, the resultant high-blebbing bacterium is a "super blebber" that shows extensive membrane blebbing in all culture conditions. Unlike virulent, high-blebbing clinical isolates of *Neisseria*, these super blebber mutants, termed *bmr,* are stable and do not revert to non-blebbing strains on repeated culture. For this reason, they are particularly useful as controls to estimate the virulence of clinical samples, for the production of neisserial membranes for use as a vaccine and for screening of antibiotics targeted at virulent *Neisseria.*

Proteins unique to the super blebbers have been identified and are especially useful for rapid identification and quantitation of virulence and as targets for the screening of antibiotics.

Outer membrane proteins and LOS are known to be useful as vaccines. (See, e.g., United States Patent Number 5,902,586 issued May 11, 1999 to Jennings et al.) Because the super blebbers produce two to four times as much outer membrane as do wild type strains, they are a particularly useful source of neisseria vaccine material. Thus, a novel two-component regulatory system has been identified in *N. gonorrhoeae, N. meningitidis* and *N. cinerea*. DNA sequence analysis suggested that the system may be non-functional in laboratory strains of *Neisseria*. However, experimental mutation of the system in each of the three species resulted in increased membrane production in the mutant compared to the wild type. This correlated with an increase in outer membrane blebbing. These data suggest that outer membrane blebbing, which

is regarded as an important contributor to the pathogenesis of neisserial diseases, is regulated as part of a coordinated response to environmental cues.

The references identified in the specification are incorporated herein by reference to the extent that they supplement, explain, provide a background for or teach methodology, techniques, and/or compositions employed herein.

The following examples are included to demonstrate the preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the concept and scope of the invention. More specifically, it will be apparent that certain agents, such as DNA constructs, that are both chemically and biologically equivalent may be substituted for the agents described herein while the same or equivalent results would be achieved. It will also be apparent that the techniques are not limited by the order in which the steps are carried out. All such substitutions and modifications apparent to those skilled in the art are deemed to be within the spirit and scope of the invention as defined by the appended claims.

The present invention provides a target for control of the neisserial membrane synthesis. The present invention also provides a quantitative assay of neisserial membrane. The present invention also provides a neisserial bacterium with increased amounts of membrane, more specifically, blebs of outer membrane, which bacterium is useful as a production cell for isolation of vaccine related membrane proteins and glycolipids. The present invention also provides proteins unique to the mutant bacterium. The present invention also provides a standard with which to compare clinical isolates of *Neisseria spp.* in order to estimate the pathogenicity of such isolates. The present invention also provides a screening assay for drugs that are specifically effective against virulent strains of *Neisseria*.

The invention is illustrated by the following examples.

Example 1. Construction of the Super Blebber

A. Bacterial strains and plasmids

Wild type *Neisseria* strains used were *N. gonorrhoeae* 1291, *N. meningitidis* NMB and *N. cinerea* 601. *Neisseria* were grown on solid GC Base medium (Difco Laboratories, Detroit) supplemented with amino acids and vitamins. For growth of kantamycin resistant *Neisseria*, BHI agar was used (Difco) supplemented with 2.5% FCS. Liquid *Neisseria* cultures were grown in proteose peptone broth (proteose peptone No. 3, Difco) 15 grams per liter; soluble starch (Difco) 1 gram per liter, dibasic potassium phosphate 4 grams per liter; monobasic potassium phosphate 1 gram per liter; sodium chloride 15 grams per liter or in Morse's defined medium.

Escherichia coli strain XL1-Blue (Stratagene, LaJolla, CA) was used to maintain plasmids and was grown in liquid culture using LB broth or on solid medium using LB agar. Antibiotics were used at the following concentrations: ampicillin 100 μg/ml; kanamycin 50 μg/ml (*E. coli*) or 25 μg/ml (*Neisseria*), erythromycin 150 μg/ml (*E. coli*) or 1 μg/ml (*Neisseria*).

pBluescript (Stratagene), pUC18 (Pharmacia, Piscataway, NJ) and pCRII.I (Invitrogen, Carlsbad, CA) were used as cloning vectors. PUC4K (Pharmacia) was used as a source of kanamycin cassette. Erythromycin resistance cassette was derived from pKErmC' plasmids (Zhou, D.G. and M.A. Apicella (1996) Gene 171:133-134.)

Plasmid DNA was prepared using Qiagen plasmid DNA preparation (Qiagen Inc., Chatsworth, CA). Chromosomal DNA was prepared from plate grown bacteria which were scraped from the plate into 100 μl of phosphate buffered saline (PBS). Cells were then pelleted by spinning for one minute at top speed in a microfuge before resuspension in 250 μl of TNE (10mM Tris-HCl pH 8, 100mM NaCl, 1 mM EDTA.) 25 l of 10% SDS were added followed by 25 μl of Proteinase K (25 mg/ml). The reaction was incubated overnight at 55° C and then extracted twice with phenol and then twice with phenol/chloroform. DNA was precipitated by addition of 10 μl 3M sodium acetate pH 5.4 and 1 ml 100% ethanol, lifted from the tube on the end of a pipette tip and washed by repeated transfer to tubes containing 500 μl 70%

ethanol. Washed DNA samples were air dried before resuspension in 100 µl TE RNase.

DNA sequencing was performed at the University of Iowa DNA sequencing facility using dye terminator sequencing chemistry with AmpliTaq DNA polymerase, FS enzyme (PE Applied Biosystems, Foster City, CA). The reactions were run on and analyzed using an Applied Biosystems Model 373A stretch fluorescent automated sequencer.

PCR was performed using Boehringer Mannheim (Philadelphia) reagents according to the protocols provided. PCRDOP was performed using degenerate oligonucleotides as described by Wren et al (FEMS Microbial Letters (1992) 99:287-291). PCR products were directly cloned using the TA cloning system (Invitrogen) according to the manufacturer's instructions.

B. Results of PCR amplification and cloning in *N. gonorrhoeae*

PCR using the degenerate oligonucleotides described by Wren produced a product of the expected size (322bp) from a *N. gonorrhoeae* genomic DNA template. The product was cloned and numerous clones were analyzed through DNA sequencing. This revealed that the PCR product contained many different DNA fragments that were clearly not from two-component regulator genes, which is in contrast to the report in which the same primer sequences amplified a region of two-component systems from numerous other bacteria without this apparent non-specific effect.

A single clone of 322 bp was obtained which through analysis of its deduced amino-acid sequence clearly contained the 5' region of the DNA-binding component of a two-component regulatory system. This clone was not identical to the *pilAB* system of *N. gonorrhoeae*.

Using the 322 bp PCR product as a probe, several genomic libraries were screened, but no positive signals were observed, although a Southern blot of *N. gonorrhoeae* genomic DNA identified several restriction fragments to which the probe stringently hybridized. Attempts to clone these fragments, all of which were 4kbp or greater in size, failed. These fragments, which might have contained the entire two-component system sought, could not be cloned, suggesting that the system was toxic to *E. coli* when carried at high copy

number. However, two *Sau3*AI fragments which were identified by the Southern analysis were successfully cloned and found to contain open reading frames (ORFs) that encoded for the majority of a two-component regulator, based on their deduced amino acid sequence. Successive cloning of other small genomic fragments, containing small parts of the two-component regulator, followed by reconstruction, produced a sequence contig of 3.97 kbp. that contained the two ORFs of the putative two-component regulator and partial ORFs upstream and downstream of the system. We have named this two-component system *bmr* (for bacterial membrane regulator, see below). The upstream ORF was homologous to a hypothetical membrane protein of *Bacillus subtilis* (Genbank submission number P42308) and the downstream ORF was homologous to the cytoplasmic axial filament protein CafA, of *E. coli* (Figure 1.) *N. gonorrhoeae bmrRS* has been deposited with the American Type Culture Collection (Rockville, MD) under the accession number PTA-801.

Comparison of the deduced amino acid sequence of the putative two-component regulator to the amino acid sequences of the Genbank database revealed homology to numerous other two-component regulators. The highest degree of homology was to the BasRS system of *E. coli* and *Salmonella typhimurium* and the putative Bas homologue in *Haemophilus influenzae*. The Bas system of *E. coli* and *S. typhimurium* is involved in regulation of lipopolysaccharide (LPS) substitution by phosphate and aminoarabinose, that in the case of *S. typhimurium* is implicated in LPS changes during the course of infection. This degree of homology is frequently found in bacteria. Biochemical capabilities of the bacteria are modified and extended by the duplication and subsequent mutation of genes, resulting in gene products with considerable degrees of homology, but having different functions and characteristics, as is seen in the *bas* and *bmr* gene products.

An interesting feature of *N. gonorrhoeae bmr* was the presence of a stop-codon at the fourteenth codon position of the DNA-binding component that was predicted to terminate translation of this ORF at that Point (Figure 2.)

C. Cloning of *bmr* from other *Neisseria*

N. meningitidis is closely related to *N. gonorrhoeae* and *N. cinerea* is a non-pathogenic member of the *Neisseria* genus. Southern blots of genomic DNA from these two bacteria, using the *N. gonorrhoeae* 322 bp PCR product as a probe, identified stringently hybridizing restriction fragments in each of these organisms. Using a strategy similar to that described above, the *bmr* locus was cloned from *N. meningitidis* and *N. cinerea*. The predicted amino acid sequences of the three loci are highly homologous, strongly suggesting that the equivalent locus from the three species had been cloned (Figure 2). However, in *N. meningitidis*, a T to G substitution altered the premature TAA stop-codon of *N. gonorrhoeae* to a GAA glutamate codon. In *N. cinerea*, the codon was also for glutamate, but read GAG. However, the *N. meningitidis* locus contains a 591 bp deletion compared to both *N. gonorrhoeae* and *N. cinerea* that results in deletion of the 3' 486 bp of the putative DNA-binding component-encoding ORF, the intergenic region and the 5' 69 bp of the putative sensor-encoding ORF (Figure 1.) In addition, other nucleotide differences in the *N. meningitidis* locus immediately preceding the deletion cause frame shifts of the BmrR ORF (Figure 2.) Thus, *N. meningitidis* is not predicted to encode a BmrR protein. Although the 5' 23 codons of the sensor-encoding ORF are deleted, an alternative initiation codon may allow translation of a near-full length sensor protein in *N. meningitidis*. The *bmr* locus was amplified by PCR from numerous *N. meningitidis* strains, including several from serotypes A, C and W 135. The primers used (5' CCTGTCCGTGTGTTGCAATC 3' and 5' CCGTTCCCGTTATCCTCCAC 3') amplify the region starting 166 bp upstream of the initiating codon of the deleted BmrR ORF and ending 200 bp upstream of the termination codon of the putative BmrS ORF. All of these PCR reactions produced a band of the same size as that from strain NMB (i.e., approximately 600 bp smaller than the fragment amplified from *N. gonorrhoeae)* (data not shown) suggesting that the deletion in *bmr* is common to many, if not all, *N. meningitidis* strains. *N. meningitidis bmrRS* has been deposited with the American Type Culture Collection (Rockville, MD) under accession number PTA-800.

An *N. gonorrhoeae bmrS* insertion mutant was constructed by insertion of an erthromycin resistance cassette at the site indicated in Figure 1. An insertion mutant of *N. meningitidis* was constructed by insertion of a kanamycin resistance cassette within the sensor coding region (Figure 1.) The locus was mutated in *N. cinerea* by deletion of an internal *Bsr*GI fragment and insertion of a kanamycin resistance cassette in its place. This mutation deletes the 3' 418 bp of *bmrR* and the 5' 565 bp of *bmrS* (Figure 1).

Figure 2 shows the alignment of the amino acid sequences of the BmrR and BmrS ORFs from the three *Neisseria spp.* The GC BmrR ORF contains a stop codon (*) at the fourteenth codon. The NM BmrR sequence contains a small deletion compared to the GC and NC sequences after the 34th codon, resulting in a frameshift (denoted by the amino acid sequence written on the line above). The amino acid sequences in frame with the N-terminal NM BmrR ORF is closed by the presence of a stop codon (*). Following the frameshift, a small insertion results in the presence of the amino acids FVPLA in the NM sequence that is absent from both GC and NC. Following this is a large deletion that removes the remainder of the BmrR ORF and the N-terminal 23 codons of the BmrS ORF. Outside of this region, the three sequences are highly homologous.

Example 2. Characteristics of the Super Blebbers

A. Chemical analysis of the outer membrane of the super blebbers

The effect of the *bmr* mutation on several parameters was investigated by comparison of the *N. gonorrhoeae* wild-type and the mutant. Overnight cultures (10 ml) were killed by the addition of 0.5% phenol for two hours and outer membrane enriched samples were isolated as described by Zollinger et al., International Symposium, Hanasaari, Espoo, Finland (1991). Samples were analyzed by SDS-PAGE on a 6-15% gradient gel. LOS was isolated by phenol/chloroform/petroleum ether extraction as described by Galanos et al. Eur. J. Biochem.(1969) 9:45-249 and analyzed as described by Lee et al. J. Biol. Chem. (1995) 270:271151-9. The growth rate and outer membrane protein profile of the mutant were unaltered from the wild type.

Because of the homology of the *Neisseria* locus to the previously characterized *bas* locus of *E. coli* and *S. typhimurium* and the involvement of this system in LPS modulation in those bacteria, LOS was isolated from wild type and mutant and analyzed by mass spectrometry. No difference was found, showing that despite the homology, the system functioned differently in *Neisseria* and those other bacteria.

B. Morphology of the super blebbers

For scanning electron microscopy (SEM), samples of proteose peptone broth cultures were taken directly without centrifugation or washing. Processing included treatment with 1% osmium tetroxide prior to dehydration through a graded ethanol series, with a final clearance in hexamethyldisilane (HMDS from Polysciences, Inc. Warrington, PA). After coating with gold-palladium, the specimens were viewed on an S-4000 Hitachi scanning electron microscope at 5kV accelerating voltage.

For transmission electron microscopy, the samples were dehydrated in a graded ethanol series prior to embedment in LR White resin (Ted Pella, Inc., Redding, CA) and sectioned to approximately 85 nm thickness using an ultra-microtome. Samples were counter-stained with 5% uranyl acetate for viewing with an H-7000 Hitachi transmission electron microscope at 75kV accelerating voltage.

SEM analysis revealed that the wild type and mutant bacteria differed in two ways. Wild type *N. gonorrhoeae* were observed to be distinct diplococci with a smooth appearance (Figures 3A and 3B) However, the mutant produced large numbers of blebs. (Figures 3C and 3D) Shedding of outer-membrane particles (blebbing) is a characteristic of *Neisseria*. In these analyses the mutant bacteria were blebbing to a much higher degree than the wild type. In addition, many mutant diplococci were joined to others by membranous connections (Figures 3C and 3E). When viewed by TEM, the connections did not contain electron dense material of the cytoplasm, suggesting that they consist of hollow membrane tubes joining pairs of diplococci. (Figure 3F) . These connections were not observed with the wild type.

The morphological changes seen in the *N. meningitidis bmr* mutant are shown in Figure 4A and 4B. As observed with the mutant *N. gonorrhoeae*, these mutants were also joined by membranous connections. The *bmr* mutant of *N. cinerea* showed similar changes in morphology.

The morphological changes are shown again in Figure 5. On strain 1291 scattered small membrane blebs can be seen emerging from the surface of the membrane. This is typical of *Neisseria gonorrhoeae* strains. The scanning electron micrograph of the superblebber *N. gonorrhoeae* 1291 *bmrS* shows extensive membrane blebbing due to mutation in the *bmrS* gene.

C. Phospholipid Analysis

The electron micrographs suggested that the mutants had alterations in their membranes that resulted in an increase in membrane material. To test this hypothesis, wild type and mutant *N. gonorrhoeae* were grown in the presence of ^{14}C-labeled acetate to label phospholipid and allow quantitation of membrane produced by the bacteria.

Bacteria were grown in 5 ml of Morse's defined medium supplemented with 4μCi/ml ^{14}C-labeled acetate. *N. meningitidis* was grown for 5 hours and *N. gonorrhoeae* was grown for 20 hours at 37°C in a shaking incubator to achieve the appropriate bacterial growth. For *N. gonorrhoeae,* the blebs were separated from the bacteria by underlaying the cultures with 2.5 ml of 10% sucrose in balanced salt buffer in centrifuge tubes and centrifuging in a SW-40 Ti rotor (Beckman, Palo Alto, CA) at 10,000 rpm at 4°C for 30 minutes. The supernatant above the sucrose cushion was transferred to fresh centrifuge tubes. The sucrose cushion was removed for scintillation counting. The supernatant was centrifuged in a SW-40 Ti rotor at 25,000 rpm (100,000 x g) for 75 minutes at 4°C to pellet blebs. The supernatant from this spin was removed for scintillation counting. The centrifuge tube was partially dried using cotton swabs.

Labeled phospholipids were isolated by extraction with 100 μl of chloroform and 200 μl of methanol added to the sample and vortexed for 2 minutes. A further 100 μl of chloroform was added followed by 30 seconds of vortexing. 100 μl of deionized water was added followed by 30 seconds of

vortexing. The mixture was centrifuged at 13,000 rpm in a microfuge for ten minutes at room temperature. The upper layer was removed for scintillation counting. The lower layer was removed from below the interface pellet and centrifuged before scintillation counting.

Blebs were separated from whole cells and the phospholipids in both samples extracted. The counts present in both bleb and cell-associated phospholipids are presented in Table I.

TABLE I

^{14}C-acetate Incorporation, Counts per Minute
N. gonorrhoeae strain 1291, wild type and *bmr* mutant

Wild Type	Experiment 1	Experiment 2	Experiment 3
Total CPM	7.4×10^7	5.7×10^7	7.1×10^7
CPM in cell-associated phospholipids	5.7×10^6	4×10^6	4.8×10^6
CPM in bleb-associated phospholipids	2.5×10^4	8.1×10^4	5×10^4
Mutant			
Total CPM	6.8×10^7	6.7×10^7	6.8×10^7
CPM in cell-associated phospholipids	7.3×10^6	4.8×10^6	5.6×10^6
CPM in bleb-associated phospholipids	5.2×10^4	3.1×10^5	2.1×10^5
Ratio of mutant to wild type cell-associated CPM	1.3	1.2	1.2
Ratio of mutant to wild type bleb-associated CPM	2.1	3.8	4.2

In each of these three experiments, *N. gonorrhoeae* 1291 *bmr* incorporated increased amounts of label compared to wild type. Both cell-associated and bleb-associated label was increased in the mutants. The increase in cell-associated counts was slight, which agrees with microscopy observations in which the mutant and wild type bacteria did not appear different except for

the presence of connections between mutant diplococci and increased level of blebbing in the mutant. As would be expected from the morphology, mutant bleb-associated counts were between two- and four-fold higher than wild type.

Labeling experiments were also conducted using *N. meningitidis*. For unknown reasons, it proved difficult to obtain consistent bleb-associated counts with these bacteria. The micrographs suggested that the membrane alteration observed with these mutants did not involve the type of blebbing seen in the gonococcus, but instead involved cell-associated membrane changes. However, total membrane was increased as was seen in the *N. gonorrhoeae* experiments. Table II shows a comparison of total outer membrane counts from both wild type and mutant bacteria. Increased incorporation of counts in the mutants was observed.

TABLE II

^{14}C-acetate Incorporation, Counts per Minute

Wild type and *bmr* mutant of *N. meningitidis* NMB

Wild-Type	Experiment 1	Experiment 2
Total CPM	4.0×10^8	6.0×10^7
Total phospholipids	1.0×10^7	5.4×10^6
Mutant		
Total CPM	4.0×10^8	6.0×10^7
Total phospholipids	1.9×10^7	9.7×10^6
Ratio of mutant to wild-type CPM	1.9	1.8

When larger quantities of membrane are desired, the culturing and purification may be easily scaled up.

Example 4. Use of the *Neisseria bmr* Mutants to Estimate Virulence of Bacteria Recovered from Clinical Samples.

Swabs from suspected *Neisseria* infections are cultured for meningococcus or gonococcus by the methods disclosed in Example 1. A laboratory strain of *Neisseria* and a super blebber are grown in the same

manner as controls. Overnight cultures are examined for blebbing in the SEM as described in Example 2B.

Phospholipids are isolated as described in Example 1. Phospholipids are quantitated as described by the incorporation of radionuclide or by HPLC analysis of total phospholipids. A level of phospholipid close to that of the super blebber indicates infection with a virulent neisserial pathogen, indicating that therapy should be immediate and aggressive.

As seen in Table III, a quantitative estimate on a scale of 1 (no blebbing) to 3 (many blebs and filamentous connections between cells) and the phospholipid content that correlates with blebbing are made to determine virulence of the clinical sample.

TABLE III

	Blebbing	Phospholipid CPM*	Diagnosis
N. gonorrhoeae strain 1291	1	6×10^4	
Super blebber	3	2×10^5	
Hypothetical Sample #1	1	6×10^4	Not virulent, therapy not needed
Hypothetical Sample #2	2	1×10^5	Moderate virulence, therapy indicated
Hypothetical Sample #3	3	$>1.8 \times 10^5$	Highly virulent, immediate and aggressive therapy indicated

*Phospholipid content is expressed as counts per minute ^{14}C-acetate incorporation into phospholipid fraction.

Example 5. Use of super blebbers to screen biocidal or biostatic agents.

A ninety-six well culture is set up with *N. gonorrhoeae* Strain 1291 and the *N. gonorrhoeae* super blebber, and the *N. meningitidis* strain NMB and the *N. meningitidis* super blebber. Dilutions of the potentially selective antibiotics are added to respective wells. An antibiotic is considered selective if it inhibits the mutant organisms that show excessive blebbing as indicated by no growth in

the super blebber chambers at lower concentrations than those that inhibit growth in the strains 1291 and/or NMB.

An compound may affect a specific property of the bacterium without outright killing of the organism. Such a compound may be particularly useful in that it does not disturb the normal microflora of a patient while inhibiting the specific property that renders a pathogen virulent. It would be particularly useful to identify a compound that targets the *Neisseria* blebbing process. A ninety-six well culture is set up with a recently isolated, high-blebbing *Neisseria* that shows excessive outer membrane formation as indicated by blebbing. Dilutions of the potentially selective antibiotics are added to respective wells. An antibiotic is considered selective if it inhibits the blebbing at lower concentrations that those that inhibit growth. Such an antibiotic may be considered to have converted the high- blebbing, virulent bacterium to a non-virulent form.

Example 6. Proteins Under the Control of the *bmr* System

The wild type *N. gonorrhoeae* strain 1291 and the isogenic mutant *bmr* strain were grown in the defined medium of Example 2, containing 40mM PO_4 ^{35}S methionine. The bacteria were harvested, washed and lyophilized as previously described. Forty milligram samples each of wild type and mutant cultures were lysed and electrophoresed in a two-dimensional gel system. The first dimension was isoelectric focusing. The final pH range was from pH 4 to pH 8. The second dimension was 14% sodium docdecyl sulfate-polyacrylamide gel electrophoresis with a molecular weight range from 200 kiloDaltons to 10 kiloDaltons. Autoradiographs of the gels from each were obtained. (Figure 6.) These were compared using a Visage Bio image 2D Analyzer Version 6.01 running on a Sun Microsystem 2.6 computer. Approximately 70 proteins were found to be under the control of the *bmr* system, that is, found in the mutant but not in the wild type.

The arrows point to several proteins that are unique to each sample.

In order to further characterize the proteins under the control of the *bmrS* two-component regulatory system, a proteomic approach was taken. To accomplish this, an Investogator 2-D Electrophoresis system (Genomic

Solutions, Charlottesville, VA) was used to analyze whole bacterial lysates. Bacteria were grown in Morse's Defined Medium. Forty micrograms of bacerial lysate were loaded onto 20 cm IEF tube gels pH gradient 4 to 8 or 3 to 10 and resolved at 18,000 volt hours. Tube gels were extruded and layered onto 22 x 22 cm 11.5% T, 2.7% C SDS-PAGE gels. These gels were stained with Coomassie Blure. Ten spots that increased in intensity in the *N. gonorrhoeae* strain *bmr S* were selected for study.

Analysis to determine the nature of the protein was performed by excising the selected spots for Coomassie Blue stained gels. The acrylamide spots were macerated, destained and the proteins digested *in situ* with trypsin. The proteolytic fragments were extracted, lyophilized and resuspended in a saturated solution of α-cyano-4-hydroxycinnamic acid. This was evaporated to leave crystalline spots on the MALDI target. Analysis was performed on a STR MALDI-TOF spectrometer (Applied Biosystems, Framingham, MA) and the spectra of proteolytic fragments compared to virtual tryptic digests of several data bases using the program MS Fit from Protein Prospector.

MS Fit is a program that is part of the Protein Prospector suite of analysis programs designed to compare trypsin proteolytic peptides to peptide fragment patterns deduced from ORFs of known genomic DNA sequences. Several protein matches are suggested and the operator then uses this data and the peptide chromatograms to judge the validity of the hits. Gene identities were determined and nucleotide sequences obtained for the Sanger or TIGR (Bethesda, MD) *N. meningitidis* genomes. These were compared to the *N. gonorrhoeae* strain FA 1090 genomic database. PCR primers were constructed from the FA 1090 sequence and used to amplify fragments of the strain 1291 genomic DNA.

These proteins were found to be coded by or under the control of the *bmrS* sequence:

atpC ATP synthase F1, epsilon subunit (MW) 15031.25 Da, pI: 5.94*

eftB electron transfer flavoprotein, beta subunit (MW:26947 Da, pI 6.08)

gpm phosphoglycerate mutase (MW: 25958.72 Da, pI 5.59)

minD septum site-determining protein (MW 29558.72 Da, pI 5.70)

nusG transcription antitermination protein (MW:20550.35 Da, pI 6.03)
*MW and pI are calculated from the TIGR NMB database.

Three of these proteins are involved in cell division and associated with membrane assembly. These are *gmp* (phosphogycerate mutase), *minD* (septum-site determining protein), and *nusG* (transcription antitermination protein).

Any of the unique proteins can serve as a marker of virulence. The proteins can be identified by techniques well known to those skilled in the microbiological art, such as immunofluorescence. In this technique, antibodies are raised against the protein of interest, either in such animals as rabbits or by the creation of hybridomas. The antibodies are conjugated to a fluorescent compound. The conjugate is allowed to contact the bacterial cells, the unbound conjugate washed away and the degree of fluorescence is measured. The bacterial cells of the sample can be those collected on a swab from the patient suspected of having a virulent infection. This is a more rapid test than that of Example 4.

Surprisingly, two of the proteins are enzymes: *atpC* (ATP synthase) and *etfB* (electron transfer flavoprotein). It is well known to those of skill in the art of enzymology to measure the level of activity of either of these two enzymes. A rise in level over that of the control *Neisseria,* such as FA 1090, will indicate increased virulence.

Any of these proteins may be used to screen neisserial biocide or biostatic agents. An agent that cause the decrease in any of these proteins is a candidate for a anti-neisserial drug.

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the previous disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be readily appreciated by those of skill in the art that many variations and modifications may be applied to the compositions and methods and used in the steps or in the sequence of steps of the methods described herein without departing from the concept and scope of the invention. More specifically, it will be apparent that certain agents, such as vectors, primers and bacterial strains, that are both chemically and

physiologically equivalent may be substituted for the agents described herein while the same or similar results would be achieved. All such similar variations and modifications apparent to those of skill in the art are deemed to be within the scope and concept of the invention defined by the appended claims.

5

We claim:

1. A mutant bacterium charcterized by having an increase in the quantity of outer membrane blebs throughout the growth cycle of said mutant bacterium during and beyond the log phase of growth and on subculturing, as compared to the wild type from which it was derived.

2. The mutant bacterium of claim 1 comprising a *bmr* locus.

3. The mutant bacterium of claim 1 which is *Neisseria gonorrhoeae.*

4. The mutant bacterium of claim 1 which is *Neisseria meningitidis.*

5. A purified protein isolated from the mutant bacterium of claim 1 which is *atpC, etfB, gpm, minD or nusG.*

6. A method of determining the virulence of bacteria in a clinical sample taken from a patient suspected of having a virulent *Neisseria* infection comprising:

(a) culturing the bacterium of claim 1, the wild type *Neisseria* from which it was derived and said clinical sample taken from a patient suspected of having a *Neisseria* infection; and

(b) examining the extent of blebbing of each bacterium; and

(c) comparing the extent of blebbing of the clinical sample to that of the bacterium of claim 1 and to that of the wild type *Neisseria* from which it was derived, thereby determining the virulence of the *Neisseria* in the clinical sample.

7. A method of determining the virulence of bacteria in a clinical sample taken from a patient suspected of having a neisserial infection comprising preparing a sample of said clinical sample suitable for two-dimensional electrophoresis and determining the presence of at least one of the proteins of claim 5.

8. A method of screening biocidal or biostatic agents that inhibit the virulence of neisserial infections, said method comprising exposing the mutant bacterium of claim 1 to said agent, and comparing the level of blebbing to that of the mutant bacterium that has not been exposed to said agent.

9. A method of screening biocidal or biostatis agents that inhibit the virulence of neisserial infections, said method comprising exposing the mutant bacterium of claim 1 to said agent and comparing the levels of any of the proteins of claim 5 to those levels of a mutant bacterium that has not been exposed to said agent.

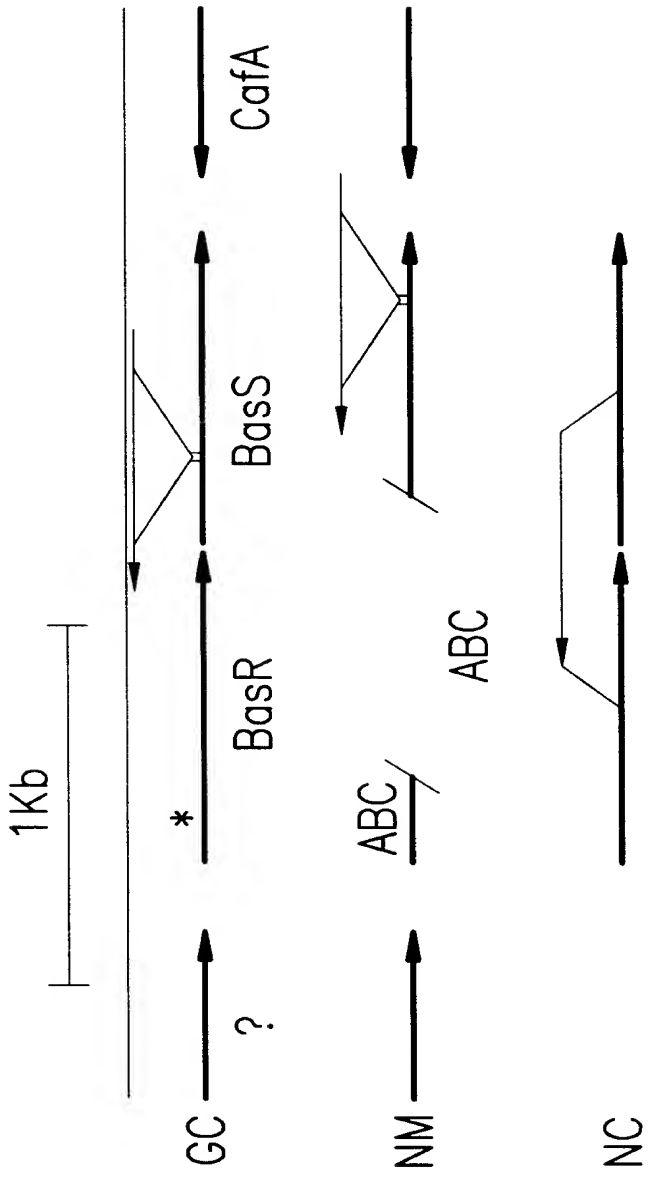

FIG. 1

```
BASR
                                                                AAA AAQPYDLMLL
NM: MRVLLVEDDA MIAEAVSASL KDGGYAVDWV KNGARLPLPS LMT*.....
GC: MRVLLVEDDA MIA*AVSASL KDGGYAVDWV KNGAQVAAAA AAQPYDLMLL
NC: MRVLLVEDDA MIAEAVSANL KDGGYAVDWV KNGAQVAAAA AAQPYDLMLL

    DLGLLGRDGL DV
NM:              MFVPL(BasS)...
GC: DLGLPGRDGL DVLSEIRAAG CTVPVLIVTA RDDLYSRLNG LDGGADDYIV
NC: DLGLPGRDGL DVLSEIRAAG CTVPVLIVTA RDDLYSRLNG LDGGADDYIV

GC: KPFDMAEFKA RMRAVLRRGS GQAQACLSNG ALSLNPATYQ VEIIAEGRQV
NC: KPFDMAELKA RMRAVLRRGS GQAQACLSNG ALSLNPATYQ VEIIAERRQV

GC: ALSNQEFSVL QALLARPGVI LSRSDSEDKV YGWGGEVESN AVDFLIHGLC
NC: ALSNKEFAVL QALLARPGVI LSRSDLEDKI YGWGAEVESN AVDFLIHALR

GC: KKLGKESIQN VRGVGWLMPR QDAV*
NC: KKLGKENIQN VRGVGWLVPG AV*

BASS
NM:                                MFVPLAM LAGMFSYYET FHETEALQDD
GC: MPDRFFKILK HSLQVRISLA LIWMFVPLAM LAGMFSYYET FHETEALQDD
NC: MLNRLIRMLK QSLQVRICIA LILMFLPLAM VAGVFSYYDT FHEAEELQDD

NM: LLRQAALYVA PDSKPETLPE GDGDTRIFVQ MPQQEDPVVS LPAHLADGLH
GC: LLRQAALYVG PDSKSETLPE GDGDTRILVQ MPQQEDPVVS LPAHLADGLH
NC: LLRQTALYVG PDYHPDALPE GDGDTRILVQ MPDQE.PIVS LPMHLKDGLH

NM: TLQADGDDDY YRVYIRTTEQ GRIAVMQENE YREDLAADAA RQSVLPLLAA
GC: TLRADGDDDY YRAYIRTTEQ GRIAVMQENE YREDLAEDAA RQSVLPLLAA
NC: TLRADEDDDY YRVYIRTTGR GRIAVMQENE YREDLAADAA MQSVLPLLAA

NM: LPLMILLTVW ITHKAMRPVR KLSQSLEQRR INDLSALSVD NIPSEIRGFV
GC: LPLMILLTVW ITHKAMRPVR KLSQSLEQRR INGLPALSVY NIPSEIRGFV
NC: LPLIILLTVW ITHQAMRPVR ILSQNLEQRR LDDLSALNTD NIPSEIKGFV

NM: TAINLLLKRA DEDIRHRQRF VADAAHELRT PMTALSLQAE RLNNMSLPPD
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NM: AARQPAVLQQ SIRRNKHLLE QLLALARSQS DETPLTKTTF GLQSRFRQVL
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NC: AARQSAVLQQ SIRRNKHLLE QLLALARSQS DETPLTKTTF GLQSRFRRVL

NM: QELMPLALEK RQDIGVAVGG DVEVSADETE IYTLIKTFAD NAVRYTPPEG
GC: QELMPLVLEK RQDIGVAVGG DVEVSADETE IYTLVKTFAD NAVRYTPNGG
NC: QELMPLALEK RQDIGVAVEG DFEIFADETE IYTLVKTFVD NAIRYTPSEG

NM: RIDLGFTDEG KYLAVWVEDN GNGIPESERA RVLDPFYRIL GTEQQGTGLG
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FIG. 2

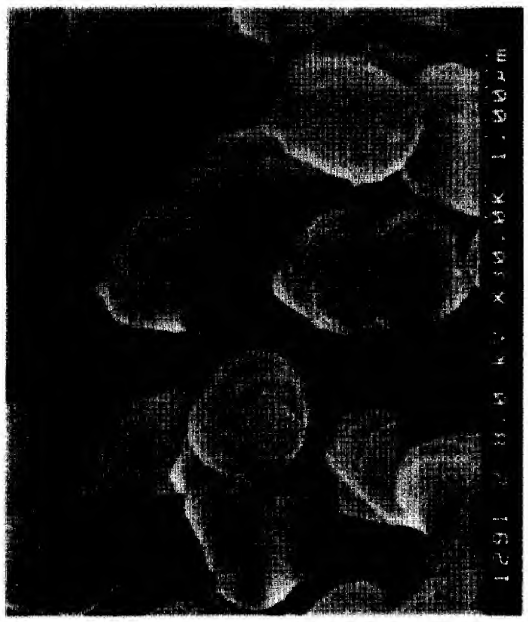


FIG. 3B

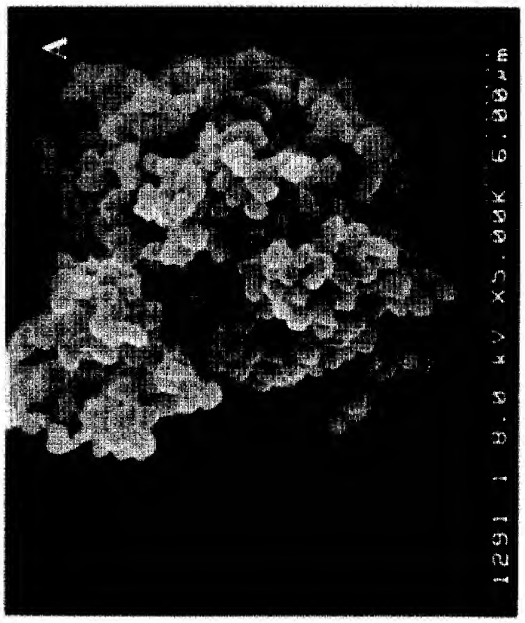


FIG. 3A

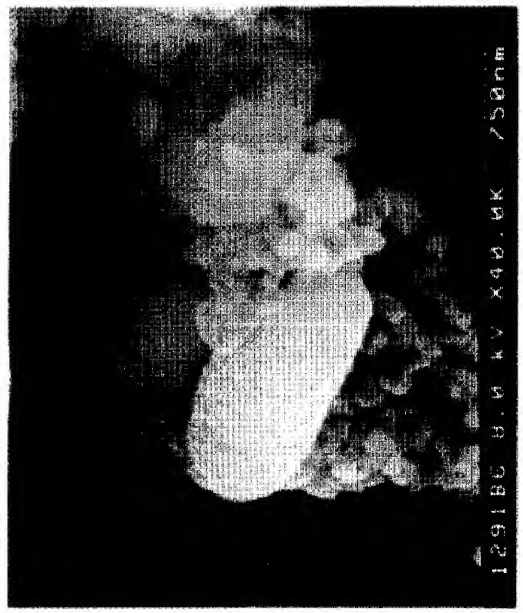


FIG. 3D

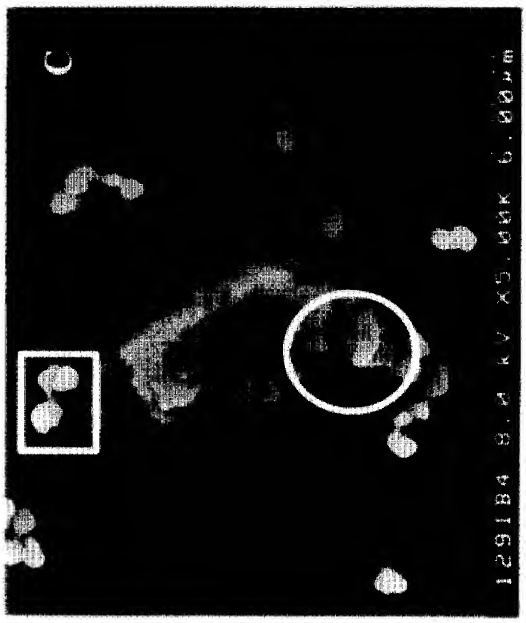


FIG. 3C

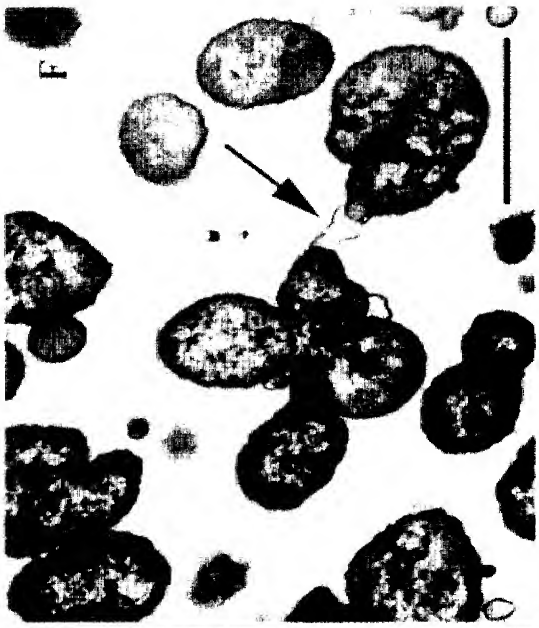


FIG. 3F

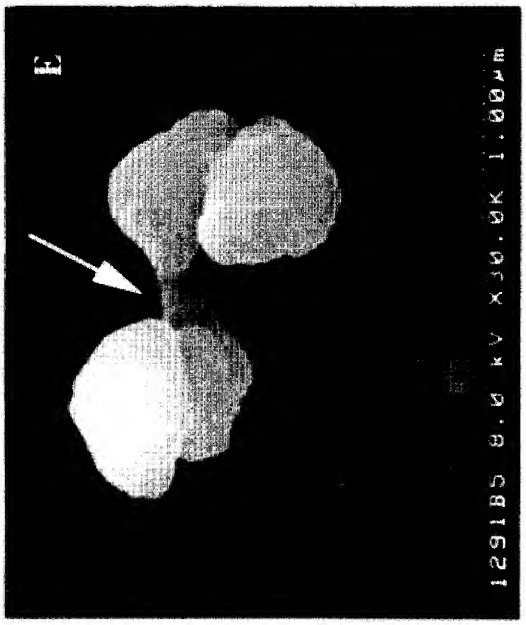


FIG. 3E

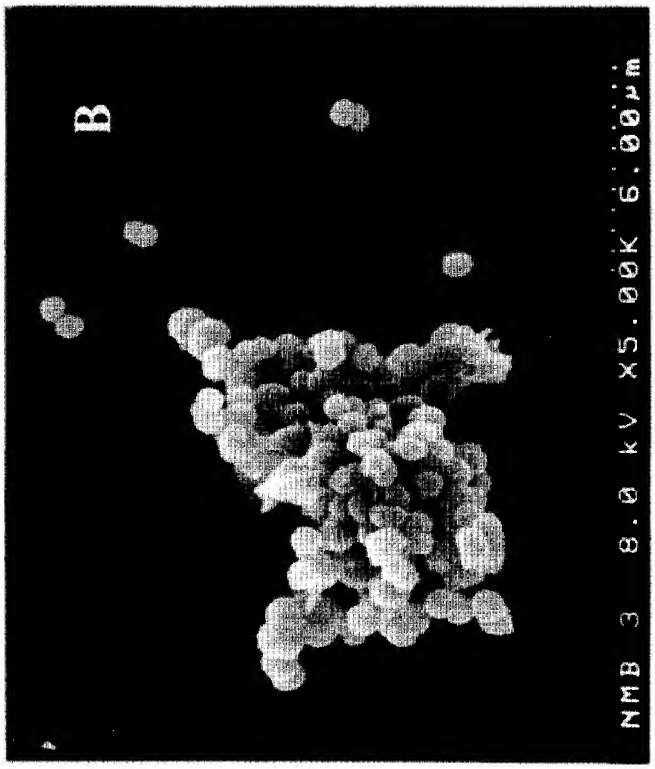


FIG. 4B

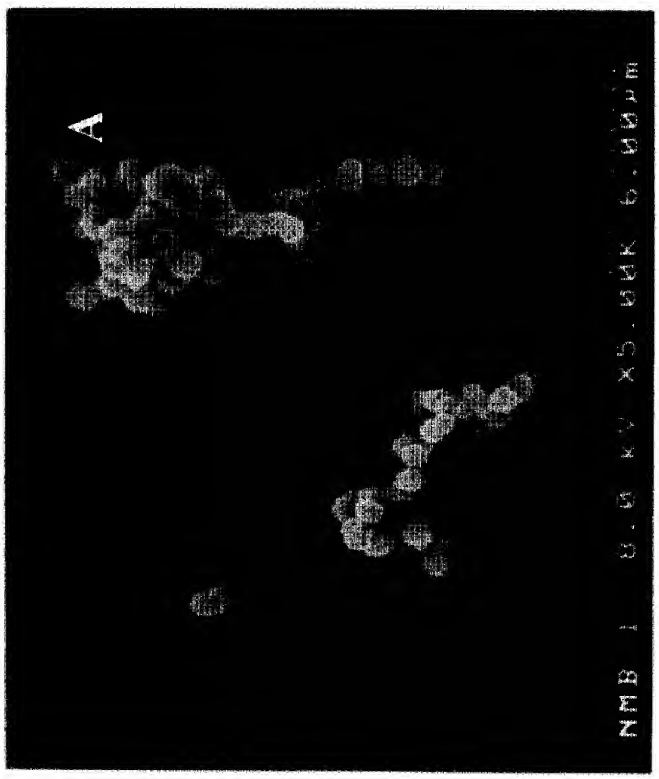


FIG. 4A

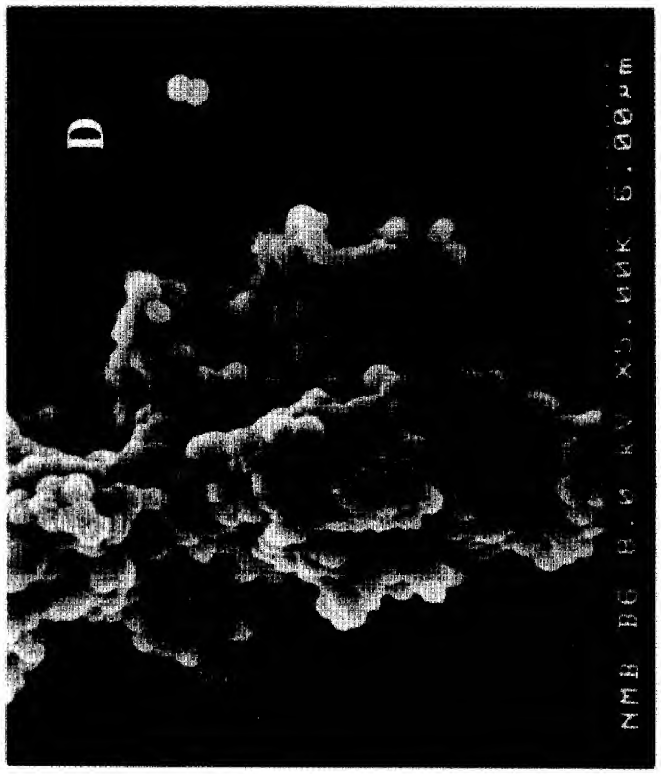


FIG. 4D

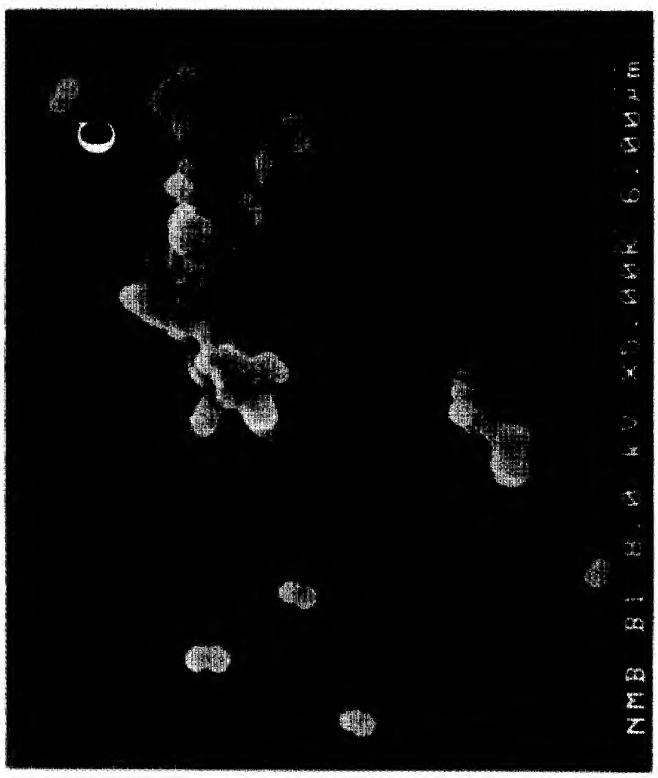
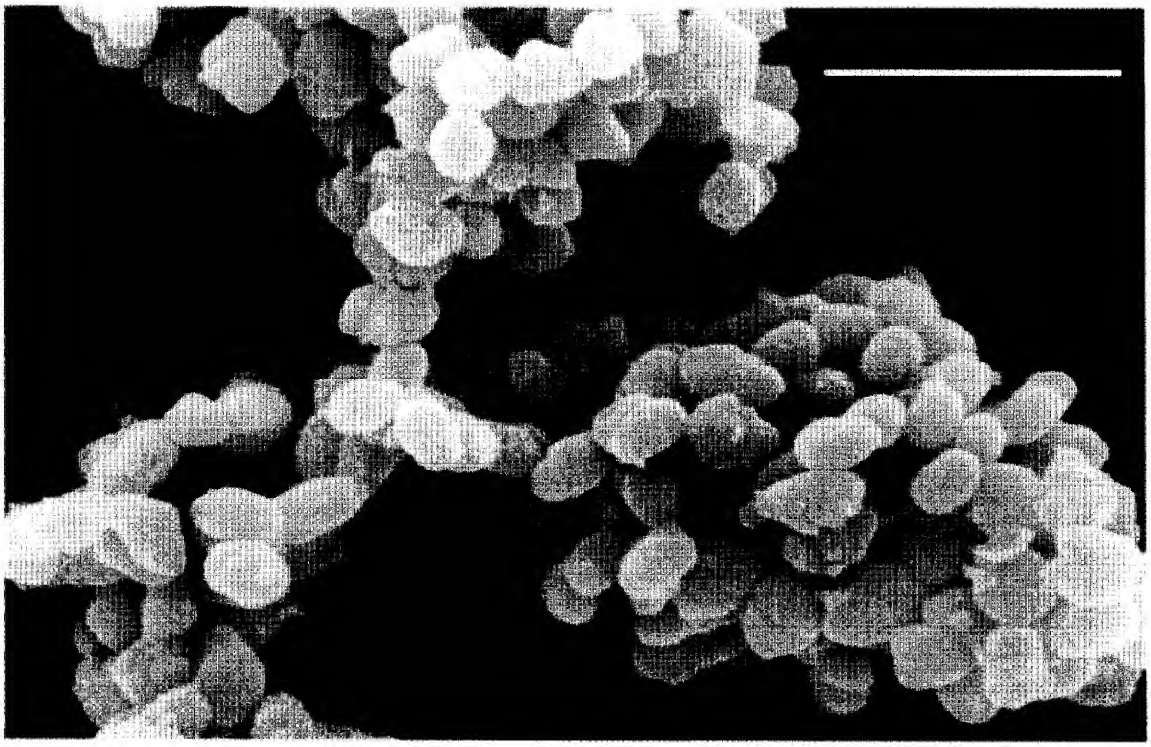
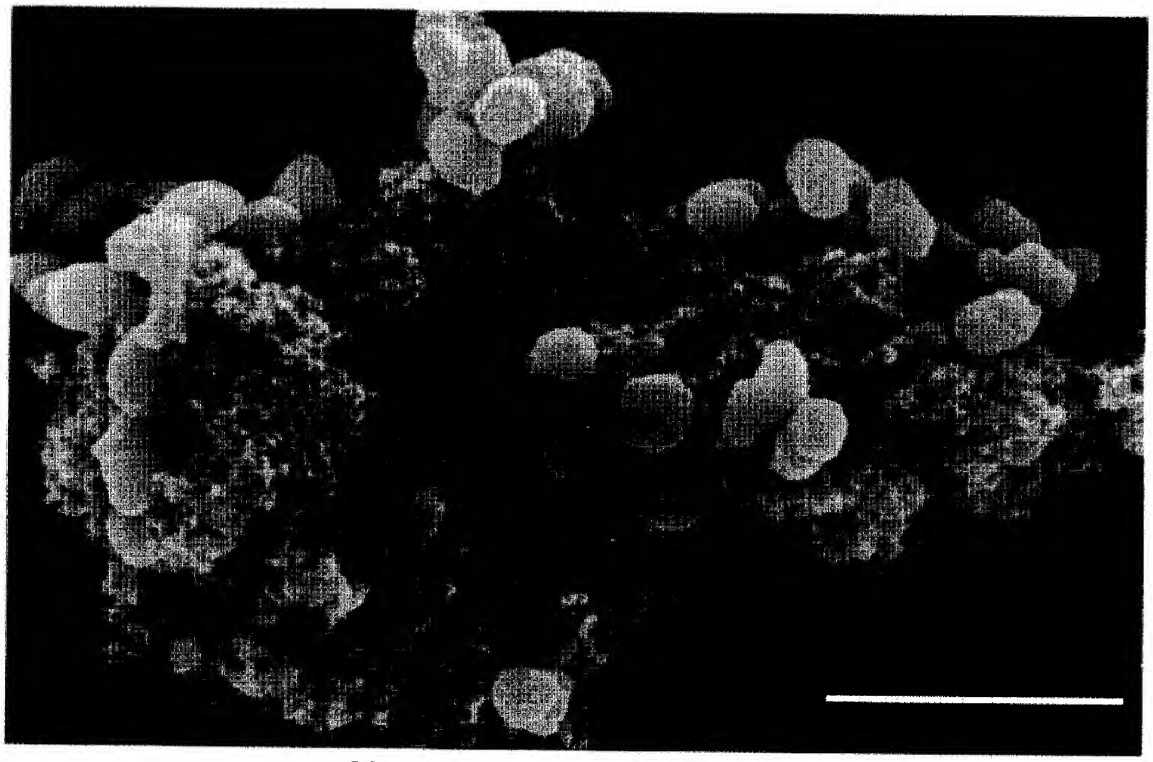


FIG. 4C



N. gonorrhoeae **1291**

FIG. 5A



***N. gonorrhoeae* 1291 bmrS**

FIG. 5B

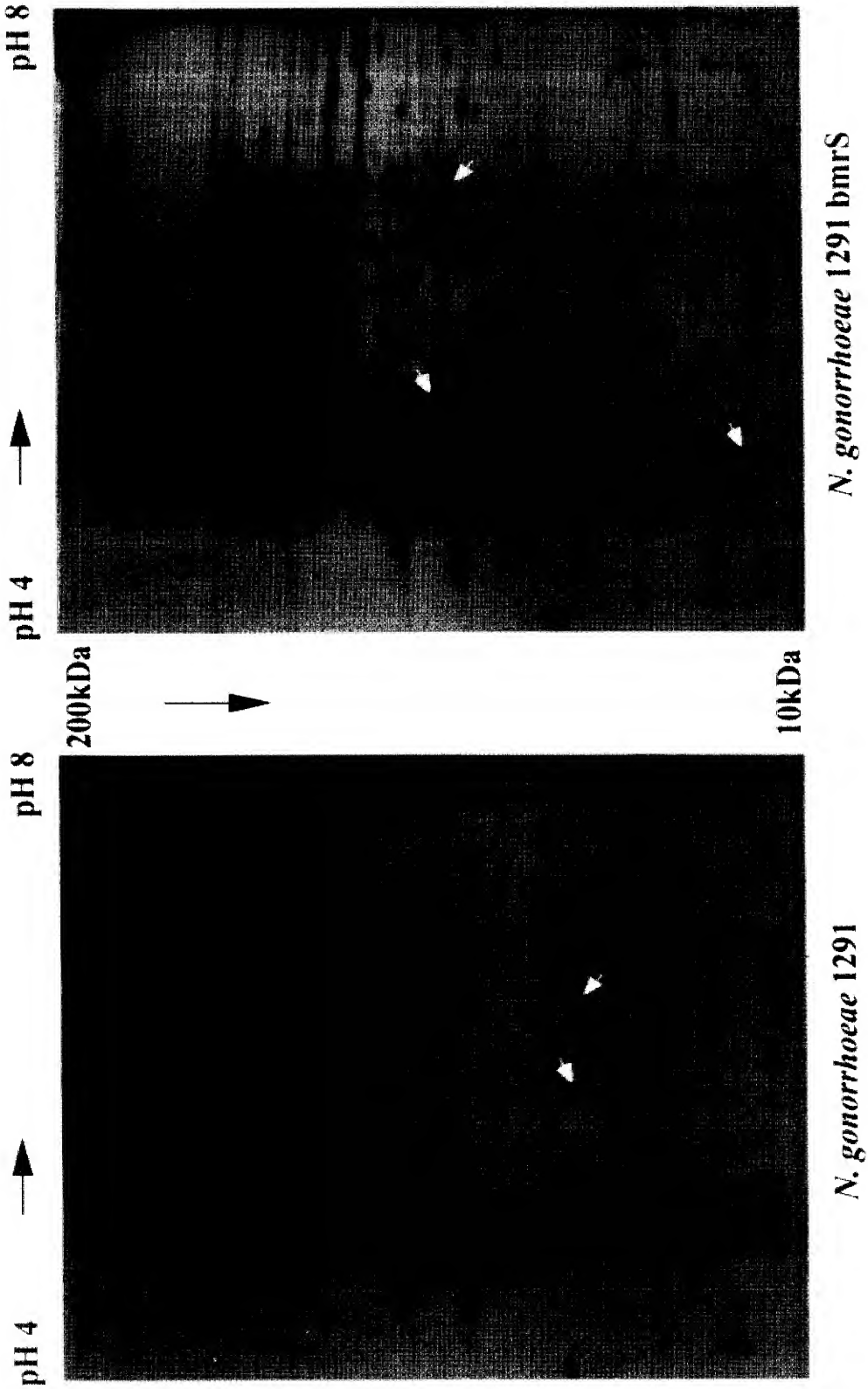

pH 8
pH 4
200kDa
10kDa
pH 8
pH 4
N. gonorrhoeae 1291 bmrS
N. gonorrhoeae 1291

FIG. 6

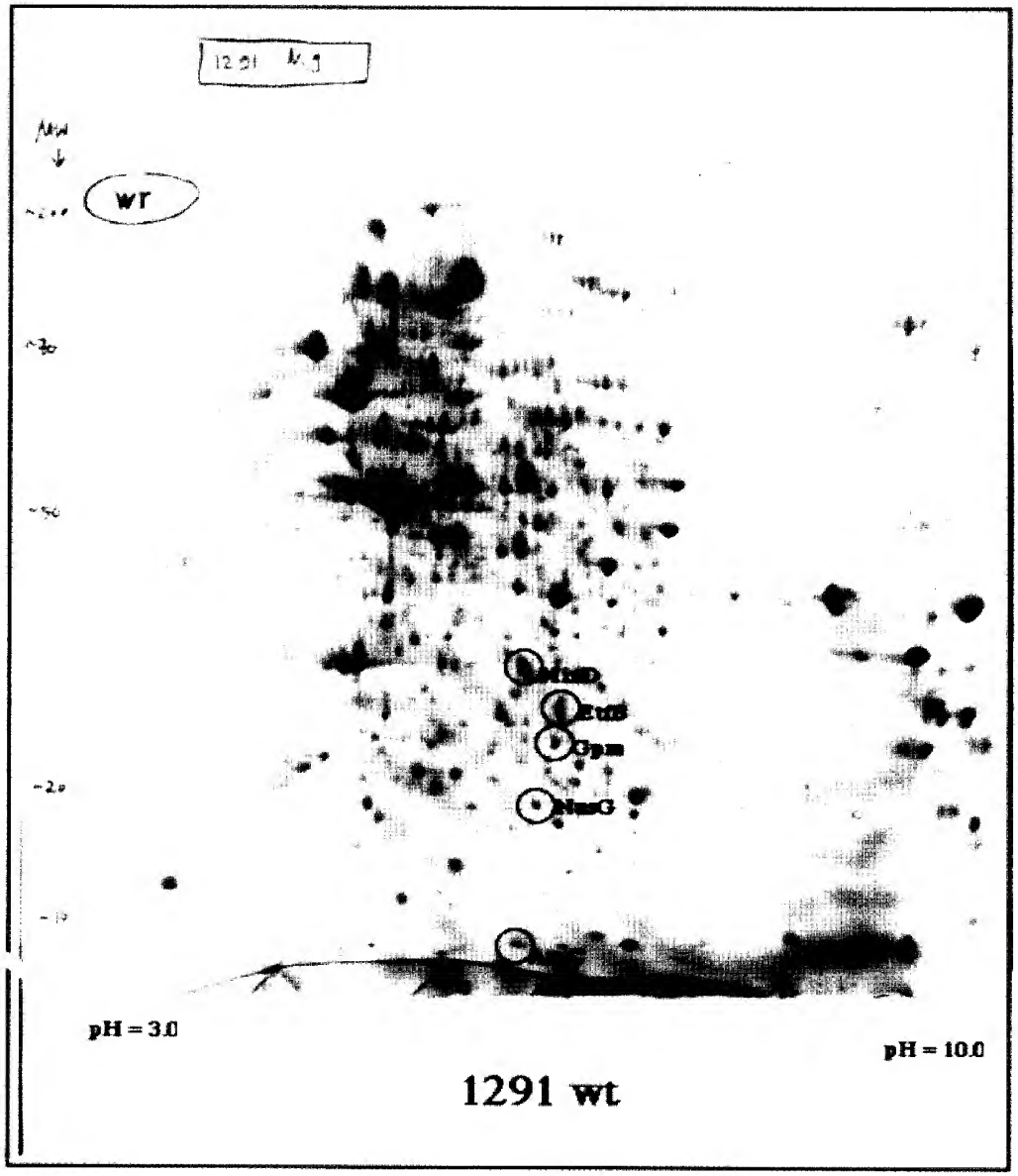


FIG. 7